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Abstract: Glioblastoma is the most common and aggressive intrinsic brain tumor in adults. Self-renewing, highly tumorigenic glioma-initiating cells (GIC) have been linked to glioma invasive properties, immunomodulation, and increased angiogenesis, leading to resistance to therapy. TGF- signaling has been associated with the tumorigenic activity of GIC. TGF- is synthesized as a precursor molecule and proteolytically processed to the mature form by members of the family of the proprotein convertases subtilisin/kexin. In this study we report that furin is unique among the proprotein convertases subtilisin/kexin in being highly expressed in human GIC. Furin cleaves and promotes activation of pro-TGF- 1 and pro-TGF- 2, and TGF- 2 in turn increases furin levels. Notably, TGF- 2 controls furin activity in an ALK-5-dependent manner involving the ERK/MAPK pathway. We thus uncover a role of ERK1 in the regulation of furin activity by supporting a self-sustaining loop for high TGF- activity in GIC.

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Extracellular signal-regulated kinase 1 (ERK1) mediates the autocrine positive feedback loop of TGF-β and furin in glioma-initiating cells

Running title: A TGF-β-furin-ERK1 autocrine loop in glioblastoma

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ABSTRACT

Glioblastoma is the most common and aggressive intrinsic brain tumor in adults. Self-renewing, highly tumorigenic glioma-initiating cells (GIC) have been linked to glioma invasive properties, immunomodulation and increased angiogenesis leading to resistance to therapy. TGF-β signaling has been associated with the tumorigenic activity of GIC. TGF-β is synthesized as a precursor molecule and proteolytically processed to the mature form by members of the family of the proprotein convertases subtilisin/kexin (PCSK). Here we report that furin is unique among the PCSK in being highly expressed in human GIC. Furin cleaves and promotes activation of pro-TGF-β1 and pro-TGF-β2, and TGF-β2 in turn increases furin levels. Notably, TGF-β2 controls furin activity in an ALK-5-dependent manner involving the ERK/MAPK pathway. We thus uncover a role of ERK1 in the regulation of furin activity by supporting a self-sustaining loop for high TGF-β activity in GIC.

INTRODUCTION

Glioblastoma is the most common malignant primary brain tumor and largely refractory to current therapies (1). TGF-β has long been attributed a major role in the malignant phenotype of glioblastoma (2, 3). Cytostatic responses to TGF-β are selectively inhibited in glioblastoma cells (4) with TGF-β sustaining the migratory and invasive potential as well as suppressing anti-tumor immune surveillance. Glioma-initiating cells (GIC) have been proposed as the cell population responsible for tumor initiation and recurrence of gliomas (5) and have been attributed immunosuppressive properties (6, 7). TGF-β may maintain the stem cell-like properties and tumorigenic activity of GIC (8-11). TGF-β binding to the TGF-β receptor complex leads to the activation of both SMAD transcription factors (canonical/SMAD-dependent pathway) (12) and various SMAD-independent pathways including the phospho-inositide 3' kinase (PI3K)/Akt pathway (13), the MEK (MAPK)/ERK, and the p38 and JNK pathways (14). Persistent activation of the TGF-β pathway in glioblastoma has been described as an autocrine loop involving TGF-β2, CREB1, SMAD3 and the PI3K/AKT pathway (2). Processing of pro-TGF-β1 and pro-TGF-β2 in the cytoplasm or in the extracellular matrix in glioma cells involves pro-protein convertases (PCSK) (15). PCSK are a class of nine enzymes with a fundamental role in the processing of diverse protein precursors (16). PCSK3, generally known as furin or paired basic amino acid-cleaving enzyme, is widely expressed and responsible for most of the processing events in the constitutive secretory pathway. Furin is located in a variety of cellular compartments including endoplasmatic reticulum, trans-Golgi network and cell surface (17). It is also considered the major endoprotease responsible for processing TGF-β at its (R/K)-2*n*X-R↓ site (where *n* = 0–3 amino acids) (18). TGF-β has been reported to induce expression of its converting enzyme furin in fibroblasts (19), epithelial cells (20), and hepatocellular carcinoma cells (21) involving SMAD and MAPK ERK1/p44 and ERK2/p42. This led us to explore the regulation of furin by these pathways in

human GIC and specifically the existence and regulation of a potential autocrine TGF-β/furin loop as a potential therapeutic target for anti-TGF-β treatment in glioblastoma.

MATERIAL AND METHODS

Cell culture and reagents

LN-308 and LN-229 kindly provided by N. de Tribolet (Lausanne, Switzerland) were cultured in DMEM (Gibco/Thermo Fisher, Madison, WI) supplemented with 1% L-glutamine (Gibco) and 10% FBS (Gibco). The human GIC T-325, T-269, ZH-161, S-24 and ZH-305 (22) were cultured in phenol red-free Neurobasal medium (NBM) (Gibco) supplemented with 2% B-27 without vitamin A (Gibco) (20 µl/ml), 1% L-glutamine (Gibco) and fibroblast growth factor and epithelial growth factor (FGF/EGF) (20 ng/ml each) (PeproTech, London, UK). Treatment of cells was performed in NBM containing 1% L-glutamine and FGF/EGF and the following reagents: PCSK inhibitor decanoyl-RVKR-CMK (3501, Tocris Bioscience, Bristol, UK), recombinant human TGF-β2 (R&D Systems, Minneapolis, MN), SD-208 (Scios, Fremont, CA) and MEK1/2 inhibitor U0126 (9903, Cell Signaling Technology, Danvers, MA).

Lentivirally-mediated gene silencing of furin was performed as described (23) using a furin specific shRNA (V3LHS_310002) cloned in a pGIPZ lentiviral vector and the respective pGIPZ vector carrying a non-targeting control (RHS4349) (Dharmacon, Lafayette, CO). Transient gene silencing was performed by electroporation (Neon Transfection System, Invitrogen/ Thermo Fisher) using 100 nM of the following ON-TARGET plus, siRNA SMART pools from Dharmacon: TGF-β1 (L-012562-00), SMAD2 (L-003561-00), SMAD3 (L-020067-00), SMAD4 (L-003902-00), ERK1 (L-003592-00), ERK2 (L-003555-00) and non-targeting control (D-001810-10).

Human PBMC were prepared from healthy donor blood and cultured in RPMI (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 20 mM L-glutamine, 50 µM β-mercaptoethanol (Sigma, St. Louis, MO), 10 U/ml penicillin (Sigma), 10 µg/ml streptomycin (Sigma), 10 mM sodium-pyruvate (Sigma), and 1x Minimum essential medium nonessential

aminoacids (Gibco). Media from GIC seeded at 10^7 in 10 ml NBM for 72 h were concentrated 2.5-fold prior to the addition to the PBMC. On day 0 PBMC were treated with 3 μg/ml concavalin A (Sigma), 20 U/ml IL-2 (PeproTech) and GIC conditioned media. At day 3 treatment with IL-2 and exposure to GIC media were repeated, and at day 6 addition of IL-2 was repeated. On day 7 cells were analyzed by flow cytometry.

Immunoblot analysis

Immunoblot analysis was performed as previously described (23). Primary antibodies were anti-furin (sc-20801, Santa Cruz Biotechnology, Dallas, TX), anti-β-actin (sc-1616, Santa Cruz), anti-human LAP/TGF-β1 (AF-246-NA, R&D Systems), anti-TGF-β1 (G1221, Promega, Madison, WI), anti-TGF-β2 (ab36495, Abcam, Cambridge, UK) and antibodies from Cell Signaling Technology specific for SMAD1 (9743), phosphorylated SMAD1/5 (Ser463/465) (9516), SMAD2 (3122), phosphorylated SMAD2 (pSMAD2 (Ser465/467)) (3108), SMAD3 (9513), phosphorylated SMAD3 (pSMAD3 (Ser423/425)) (9520), SMAD4 (9515), p44/42 MAPK (ERK1/2) (9102), phospho-p44/42 MAPK (ERK1/2) (9106), Akt (9272), phospho-Akt (Ser473) (9271), p38 MAPK (9212), and phospho-p38 MAPK (9211) Secondary antibodies were HRP-coupled goat anti-rabbit (sc-2004), donkey anti-goat (sc-2033) (Santa Cruz Biotechnology) or sheep anti-mouse antibodies (NA931V, Ge Healthcare UK limited, Amersham, UK).

RT-PCR

RT-PCR was performed by using the ΔC_T method with ARF1 as a housekeeping gene (see supplemental table I for primers).

Furin-specific activity

Cellular and extracellular furin-specific activity (FSA) was measured as described (24) using the anti-furin antibody MAB15032 (R&D Systems) as capturing antibody in lysates and conditioned media of cells seeded at a density of $5x10^6$ cells in 7 ml NBM supplemented with L-glutamine and FGF/EGF for 48 h. FSA is expressed as the fluorescence intensity (λexc=380nm, λem=460nm) normalized to protein concentration as determined by Bradford assay (Bio-Rad, Hercules, CA). FSA was measured in concentrated conditioned media mixed with the appropriate amount of reaction buffer and analyzed in the same manner as previously described for the lysates (24).

Flow cytometry

APC-CD25 (17-0259) and PE-FOXP3 (12-4776) antibodies were from eBioscience (San Diego, CA), FITC-CD4 (55346) from BD Biosciences (San Jose, CA). FOXP3 fixation/permeabilization kit (eBioscience) was used for FOXP3 staining. Cells were analyzed with FACS Verse (BD biosciences) and data analyzed with FlowJo software. Fluorescence Minus One (FMO) controls using fluorochrome-conjugated isotype control antibodies from BD biosciences (FITC-mouse IgG1, k (555748), APC-mouse IgG1, k (555751) and PE-rat IgG2A, k (553930)) were used.

RESULTS AND DISCUSSION

Seven of the nine PCSK, i.e. PCSK1, PCSK2, PCSK4, PCSK5, PCSK6, PCSK7 and furin, process their substrate at the consensus recognition sequence $(R/K)X_n(R/K)\downarrow$ which is also the recognition site for processing TGF-β. We first investigated mRNA expression levels of these enzymes in a panel of five human GIC: T-325, T-269, ZH-161, S-24 and ZH-305. Furin was expressed in all cell lines and one of the most abundant PCSK (Figure 1A). Furin was detectable intracellularly in cell lysates as a double band of around 100 kDa, corresponding to the two glycosylated/sialylated forms of mature furin (25) except for T-269 where only the higher molecular mass isoform was detected. In the conditioned media furin was detectable as a major band with an apparent molecular mass of around 90 kDa (Figure 1B).

To investigate the processing of TGF-β1/2 by furin, lentiviral gene silencing of furin was performed in T-325, ZH-161 and ZH-305 cells, further referred to as shfurin cells (Figure 1C-E). Furin gene silencing did not affect any other PCSK with the exception of PCSK6 in ZH-305 shfurin cells (data not shown). Decreased protein levels of both cellular and secreted furin (Figure 1C) and a reduction in cellular and extracellular furin-specific activity (FSA) (Figure 1D) were confirmed in all furin knockdown cell lines. We then analyzed TGF-β1/2 processing in shfurin cells. For TGF-β1 we used an antibody to the N-terminus of TGF-β1 detecting both pro-TGF-β1 (55kDa) and the latency associated peptide (LAP) of TGF-β1 (37 kDa) and an antibody to the C-terminus detecting mature TGF-β1 (12.5 kDa) (see ZH-161/siTGF-β1 as a control, Figure 1C). For TGF-β2 we used an antibody reacting with the C-terminus of TGF-β2 detecting both pro-TGF-β2 (55 kDa) and mature TGF-β2 (12.5 kDa). Furin gene silencing resulted in increased levels of pro-TGF-β1 and correspondingly decreased levels of the two respective processing products LAP/TGF-β1 and mature TGF-β1 in ZH-161 and ZH-305, with TGF-β1 being not detectable in T-325 (Figure 1C). In T-325 and ZH-305, showing detectable TGF-β2 protein levels, furin gene silencing increased levels of pro-TGF-β2 and

decreased levels of mature TGF-β2. The effects of furin gene silencing on pro-TGF-β1/2 processing were similar to those obtained with the pan-proprotein convertase inhibitor decanoyl-RVKR-CMK (PCSK inhibitor), suggesting that furin is the main PCSK involved in pro-TGF-β1/2 processing here. Notably, in T-325 and ZH-161 pro-TGF-β processing takes place to a remarkable extent extracellularly as pro-TGF-β levels increased in shfurin cells and upon addition of the PCSK inhibitor in the conditioned media, but remained unchanged in the lysates. In ZH-305, the levels of pro-TGF-β2 increased both in cell lysates and in conditioned media following furin gene silencing, suggesting that pro-TGF-β2 processing takes place in part in the intracellular compartment in ZH-305 cells, too (Figure 1C).

To confirm that reduced TGF-β levels affect down-stream signaling activities, we analyzed the levels of phosphorylated SMAD (pSMAD)2, SMAD3 and SMAD1/5 as readouts for the activation of canonical TGF-β signal transduction, and of phosphorylated Akt (Ser473) (pAkt (Ser473)), ERK1/2 (pERK1/2) and p38 (pp38) reflecting non-canonical TGF-β signaling (Figure 1E). All sh-furin cells showed reduced levels of pSMAD2 and pSMAD3. In ZH-161 and ZH-305 pSMAD1/5 was also reduced, pointing towards a reduction in basal bone morphogenetic proteins (BMP) signaling upon furin gene silencing, too. Indeed, inhibiting TGF-β by the TGF-β RI/activin receptor-like kinase (ALK)-5-specific inhibitor SD-208 did not decrease endogenous pSMAD1,5 levels in GIC, although the increase in pSMAD1,5 by exogenous TGF-β was blocked (26). The phosphorylation of AKT (Ser467) and p38 was reduced in T-325 shfurin cells, and ZH-305 shfurin cells showed a reduction in the phosphorylation of ERK and p38 as well.

TGF-β exerts several immunosuppressive functions, including the promotion of FOXP3+ regulatory T (Treg) cells. Previous studies have demonstrated that GIC induce Treg generation *in vitro* (6). Accordingly, we treated human PBMC with GIC conditioned media and confirmed the induction of CD4+CD25+FOXP3+ cells: conditioned media of ZH-161 cells increased the fraction of CD4+CD25+FOXP3+ cells from 8.9 to 14.8 ± 0.1%. TGF-β2

178 treatment was used as a positive control. To test whether the inhibition of TGF-β processing
179 affected the ability of GIC to induce Treg, we exposed PBMC to media derived from GIC
180 treated with the PCSK inhibitor. This led to a decrease in the induction of the
181 CD4+CD25+FOXP3+ population from 8.9 to 11.1 ± 0.5 % (Figure 1F), indicating that indeed
182 the inhibition of TGF-β processing in GIC may reduce their immunosuppressive properties.
183 Similar results were obtained for ZH-305 (data not shown).
184 Autocrine production of TGF-β may be necessary to maintain GIC stemness and TGF-β
185 signaling in the tumor microenvironment (27), however, the mechanisms how GIC maintain
186 high TGF-β activity is not well understood (2). We therefore evaluated the effect of TGF-β2
187 on furin levels. Indeed, TGF-β2 induced the expression of furin and FSA in all cell lines
188 except T-269 (Figure 2A-C). To investigate whether the expression of PCSK other than furin
189 was affected by TGF-β2, we analyzed the mRNA levels of the other six PCSK in ZH-161
190 (Figure 2D), T-325 and ZH-305 cells (data not shown) cells treated with TGF-β2. Indeed,
191 none of them was changed, pointing towards a specific effect of TGF-β2 on furin expression.
192 Concentration and time dependency of furin induction was investigated in ZH-161 cells
193 (Figure 2E-G). Since we observed an induction on protein levels after 24 h (Figure 2F) and
194 the maximum effect was achieved with 2.5 ng/ml TGF-β2 (Figure 2G), we selected these
195 conditions for future experiments. We proceeded to study the signal transduction mechanisms
196 involved in the regulation of furin by TGF-β2. Exposure of T-325, ZH-161 and ZH-305 to the
197 ALK-5 inhibitor SD-208 had no effect on constitutive furin protein levels, but abolished TGF-
198 β2-mediated furin induction (Figure 2H). The transient gene silencing of SMAD2, SMAD3 or
199 SMAD4 did not abrogate the induction of furin expression by TGF-β2, indicating
200 involvement of SMAD-independent signal transduction (Figure 2I).
201 Indeed, blocking the MEK1/2 branch of the non-canonical TGF-β pathway by U0126
202 decreased constitutive furin levels and attenuated the increase in furin levels in response to
203 TGF-β2. U0126 also decreased constitutive furin levels in T-269 cells which are the TGF-

β2/furin-non-responsive model (Figure 3A). The inhibitory effect of U0126 on the induction of furin expression by TGF-β2 translated into a reduction in FSA (Figure S1A). Similarly, the selective gene silencing of ERK1, but not of ERK2, reduced furin basal levels in all cell lines and attenuated the induction of furin by TGF-β2 (Figure 3B-C). Gene silencing of ERK1, but not of ERK2, reduced furin on mRNA levels in both basal conditions and upon TGF-β2 treatment (Figure S1B). To address whether the control of furin by ERK1 and TGF-β2 is associated with a glioma stem cell-like phenotype, we examined the same GIC cultured in differentiating conditions (Figure S1C) and the long term glioma cell lines, LN-308 and LN-229 (Figure S1D). In both cases ERK1 gene silencing reduced basal furin levels and attenuated the induction of furin by TGF-β2, suggesting that this molecular pattern is shared between cells with stemness properties and more differentiated tumor cells. The reduction in furin levels following ERK1 gene silencing was associated with a reduction in pro-TGF-β1/2 processing with increased pro-TGF-β levels and decreased mature TGF-β levels (Figure S1E). MEK1/2 inhibition did not significantly affect the number of spheres, but the spheres formed by GIC (T-325, ZH-161 and ZH-305) treated with U0126 were smaller, indicating an inhibitory effect on cell proliferation as confirmed by reduced MTT metabolism (Figure 3D). This rather implies a role for the TGF-β-ERK1-furin loop in sustaining the expansion of more differentiated, i.e. progenitor-like glioma cells, rather than in directly promoting GIC sphere formation. Targeting ERK1/2 also attenuated the immunosuppressive properties of GIC since treatment of ZH-161 and ZH-305 cells with U0126 decreased the induction of CD4+CD25+FOXP3+ cells by conditioned media of ZH-161 (Figure S1F) and ZH-305 (data not shown). Thus, our findings reveal that the MEK1/2 branch of the non-canonical signaling pathway, often deregulated in glioma stem cells (28), plays a pivotal role in the control of furin levels in GIC via ERK1. Altogether, we report that TGF-β induces furin activity in GIC and we specifically identify the ERK1 pathway as an essential pathway to maintain furin

activity. Our findings indicate that the disruption of ERK1 signaling would be a therapeutic strategy combating TGF-β activity in GIC.

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FOOTNOTES

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2. Abbreviations: FSA: furin-specific activity; GIC: glioma-initiating cells; PCSK: proprotein convertases subtilisin/kexin,. Treg cells: regulatory T cells.

FIGURE LEGENDS

FIGURE 1. Furin processes TGF-β in GIC. A. RT-PCR analysis of PCSK1, PCSK2, PCSK4, PCSK5, PCSK6, PCSK7 and furin. **B.** Analysis of furin levels by immunoblot in the lysates and conditioned media. **C.** Protein levels of furin and precursor forms of TGF-β1/2 were determined in cell lysates and conditioned media of furin-silenced or control cells or cells treated with 25 μM PCSK inhibitor decanoyl-RVKR-CMK for 48h. Precursor and mature forms of TGF-β1/2 were determined in the conditioned media of control, furin-depleted and PCSK inhibitor-treated cells. **D.** Cellular and extracellular FSA was measured in lysates and conditioned media of control or furin-depleted cells. **E.** Total SMAD1,2,3 phosphorylated SMAD1,2,3,5, total and phosphorylated AKT, ERK1/2 and p38 levels were determined in control or furin-depleted cell lysates by immunoblot. In brackets the theoretical molecular mass is indicated. **F.** Human PBMC were treated with 2 ng/ml TGF-β2 or conditioned media from untreated ZH-161 cells or ZH-161 cells treated with 25 μM PCSK inhibitor decanoyl-RVKR-CMK, in presence of IL-2, for 7 days. Cells were stained for CD4 (FITC), CD25 (APC) and FOXP3 (PE). Scatter plots are gated on CD4+. (P=0.0185 for PBMC treated with media from PSCK inhibitor-treated versus PSCK inhibitor-untreated ZH-161, unpaired Student's t-test). All experiments were performed at least twice and data are mean of triplicates ± SD.

FIGURE 2. TGF-β2 induces furin in GIC. A-B. Furin expression was analyzed on mRNA level by RT-PCR (A) and on protein level in cell lysates by immunoblot (B) in GIC treated with 2.5 ng/ml TGF-β2 for 8h (A) or 24h (B). **C.** FSA was measured in the cell lysates and in conditioned media of T-325 and ZH-161 treated with 2.5 ng/ml TGF-β2 for 48 h. **D.** PCSK1, PCSK2, PCSK4, PCSK5, PCSK6, PCSK7 and furin mRNA levels in ZH-161 treated with 2.5 ng/ml TGF-β2 for 24 h. **E.** Furin mRNA levels were measured by RT-PCR in ZH-161 treated with TGF-β2 for the indicated time points. **F.** Furin protein levels were analyzed in cell

lysates and conditioned media of ZH-161 treated with 2.5 ng/ml TGF-β2 for the indicated time points by immunoblot. **G.** ZH-161 were treated with the indicated concentrations of TGF-β2 for 24 h. Furin protein levels were analyzed in cell lysates by immunoblot. **H.** Furin levels were analyzed in the lysates of T-325, ZH-161 and ZH-305 treated with 2.5 ng/ml TGF-β2, 1 μM SD-208 or the combination of TGF-β2 and SD-208 for 24 h. DMSO was used as solvent control. **I.** T-325, ZH-161 or ZH-305 were transfected with siRNA targeting SMAD2 (left panel), SMAD3 (central panel), SMAD4 (right panel) or non-targeting control and 24 h later treated with 2.5 ng/ml TGF-β2 for 24 h. Furin was analyzed in the cell lysates by immunoblot. All experiments were performed at least twice and data are mean of triplicate ± SD.

FIGURE 3. ERK1 controls furin expression in GIC. A. Furin levels were determined in GIC pre-incubated with the MEK1/2 inhibitor U0126 (10 μM) for 1 h and then treated with 2.5 ng/ml TGF-β2 for 24 h. DMSO was used as solvent control. **B, C.** Cells were transfected with siRNA pools targeting ERK1, ERK2, the combination of ERK1 and ERK2 or non-targeting control. 48 h later T-325, ZH-161, S-24 and ZH-305 were treated with TGF-β2 for 24 h. Furin was analyzed in the cell lysates by immunoblot. **D**. Sphere formation assay in T-325, ZH-161 and ZH-305 cells treated with 10 μM U0126 or solvent control for 15 days. Data are absorbance values after MTT addition. All experiments were performed at least twice and data are mean of triplicate ± SD (* $P<0.05$, ** $p<0.01$, unpaired Student's t-test).